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(54) Title: HYDRAZINE CONTAINING CONJUGATES OF POLYPEPTIDES AND GLYCOPOLYPEPTIDES WITH POLYMERS (57) Abstract Biologically active macromolecular conjugates of a biologically active polypeptide or glycopolypeptide and one or more water-soluble polymer molecules covalently bonded thereto at a reactive carbonyl or carboxylic acid group of a peptide moiety on the polypeptide or glycopolypeptide or at an oxidized carbohydrate moiety of the glycopolypeptide by a linkage containing a hydrazide or hydrazone functional group. The linkage preferably also includes an amino acid or a peptide sequence.		

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HYDRAZINE CONTAINING CONJUGATES OF
POLYPEPTIDES AND GLYCOPOLYPEPTIDES WITH POLYMERS
Technical Field

5 The present invention relates to biologically active macromolecular conjugates, in particular, to conjugates of biologically active polypeptides and glycopolypeptides with water-soluble polymers.

Background Art

10 The conjugation of polypeptides with water-soluble polymers such as polyethylene glycol (PEG) is well known. The coupling of peptides and polypeptides to PEG and similar water-soluble polymers is disclosed by U.S. Patent No. 4,179,337 to Davis et al.

15 Davis et al. discloses that physiologically active polypeptides modified with PEG exhibit dramatically reduced immunogenicity and antigenicity. Also, the PEG-protein conjugates, when injected into a living organism, have been shown to remain in the
20 bloodstream considerably longer than the corresponding native proteins. Accordingly, a number of PEG-conjugated therapeutic proteins were developed exhibiting reduced immunogenicity and antigenicity and longer clearance times, while retaining a substantial
25 portion of the protein's physiological activity. Significant PEG-conjugated therapeutic proteins include tissue plasminogen activator, insulin, interleukin 2 and hemoglobin. Furthermore, Dreborg et al., Crit. Rev. Therap. Drug Carrier Syst., 6, 315-65 (1990) disclose
30 that covalent modification of potent allergen proteins with PEG often can be effective in reducing their allergenicity. Sehon, et al., Pharmacol. Toxicol. Proteins, 65, 205-19 (1987) disclose that such
35 PEG-conjugated allergen proteins having reduced allergenicity can then be utilized as tolerance inducers.

 In most instances, as exemplified by U.S. Patent No. 4,179,337, covalent attachment of the polymer

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is effected by reacting PEG-succinimide derivatives with amino groups on the exterior of protein molecules. However, the amino groups of many proteins are moieties responsible for polypeptide activity that can be readily inactivated as a result of such modification. The conjugation of such proteins is not desirable, because it results in the reduction of physiological activity. Other proteins may have only a small number of available amino groups, and consequently very few polymer anchoring sites. As a result, many proteins of interest cannot be conjugated with PEG in this manner.

The known alternatives to covalent attachment of polymers to other functional groups on the exterior of proteins have serious limitations. U.S. Patent No. 4,179,337 discloses, for example, that PEG-maleimide derivatives can be used to covalently attach polymers to protein sulfhydryl groups. However, this is of limited versatility because very few proteins have free sulfhydryl groups that are not required for biological or enzymatic activity and would thus be available for chemical modification.

U.S. Patent No. 4,179,337 discloses the reaction of an amino-PEG derivative with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide(EDC)-activated carboxylic acid groups of trypsin and other proteins. The selectivity of this reaction is rather poor because the reactivity of amino-PEG is similar to that of the lysyl residues of proteins, with both the amino-PEG and protein amino groups competing to react with the activated carboxylic acid groups. This results in intermolecular as well as intramolecular crosslinking and a loss of protein activity.

In a similar reaction disclosed by Pollack et al., JACS, 98, 289 (1976), p-aminobenzyl ethers of PEG are coupled to carboxylic acid groups of D-glucose-6-phosphate dehydrogenase by treatment with EDC.

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A polymer derivative that protein amino groups would not compete with for activated carboxylic acid groups of proteins would be highly desirable. This would eliminate intermolecular and intramolecular crosslinking and improve the enzymatic activity of polymer conjugates.

U.S. Patent No. 4,847,325 to Shadle et al. suggests that glycosylated Colony Stimulating Factor-1 (CSF-1) could be covalently attached to PEG by reacting PEG-amine, PEG-hydrazine or PEG-hydrazide with CSF-1 that had been oxidized with periodate to convert vicinal diols in the sugars to aldehydes. However, this disclosure is silent regarding the details on preparation of such conjugates and their reactivity.

The degree of polymer conjugation with amino groups is ordinarily determined by assaying the conjugate with trinitrobenzene sulfonic acid (TNBS) to determine the number of free amino groups. For polymers conjugated at protein amino groups, the difference between the number of free amino groups in the modified protein and the number of free amino groups in the native protein represents the degree of conjugation of the protein.

The results from TNBS assays are meaningless when determining the degree of conjugation of proteins when the polymer is covalently attached to alternative functional groups. In such instances, the number of free amino groups will not vary between conjugated and non-conjugated protein species.

The conjugated protein can also be digested in small fragments with an enzyme and separated by column chromatography followed by preparation of a peptide map for comparison to a map of the unmodified protein, with the fragments having altered elution times indicative of the location of polymer attachments. However, this procedure consumes large quantities of product and is not suitable for use with polypeptides of limited availability. Radioactive labeling presents another

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alternative, but this alternative is not suitable for materials being prepared for therapeutic end uses for which the determination of degree of conjugation is most critical.

5 Yamasaki et al., Agric. Biol. Chem., 52(8),
2125-7 (1988) disclose the preparation of
PEG-succinimide derivatives with norleucine and lysine
residues between the polymer and the succinimido moiety,
which residues permit the measurement of the amount of
10 PEG covalently attached to the amino groups of proteins
by amino acid analysis for the presence of norleucine or
lysine. Sartore et al., Proced. Intern. Sym. Control.
Rel. Bioact. Mater., 17, 208-9 (1990) also disclose the
use of a norleucine spacer in PEG-succinimide
15 derivatives covalently bonded to protein amino groups,
noting that the use of such an unnatural amino acid
helps in the characterization of the adduct because a
single amino acid analysis would give both protein
concentration and number of polymer chains bound to the
20 amino groups. In other words, in the purified
conjugate, each single norleucine residue acid
represents a polymer chain bound to an exterior amino
group.

 There remains a need for methods to covalently
25 attach polymers to non-amino moieties of polypeptides
and glycopolypeptides without a loss of activity from
intermolecular crosslinking, as well as for methods of
assaying the degree of conjugation of the polymer to the
polypeptide at functional groups other than amino
30 groups.

Summary of the Invention

 It has now been discovered that water-soluble
polymers can be conjugated with biologically active
polypeptides and glycopolypeptides utilizing acyl
35 hydrazine derivatives of the water-soluble polymers.
The acyl hydrazine derivatives of the water-soluble
polymers covalently link to either the oxidized
carbohydrate residues of the glycopolypeptides or the

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reactive carbonyl or activated carboxylic acid groups of peptide moieties of polypeptides or glycopolypeptides. This invention extends the realm of water-soluble polymer-peptide conjugation to those polypeptide and glycopolypeptide materials that could not have been modified heretofore by conventional methods. Furthermore, under neutral or mildly acidic condition of conjugation reactions, due to their low pK_a (about 3) acyl hydrazine containing polymers of this invention possess higher reactivity than the amino groups of polypeptides (pK_a about 10.5), therefore minimizing and in most cases eliminating the competing reactions of these amino groups, thus preventing polypeptide crosslinking and preserving the biological activity of the conjugates.

In accordance with the present invention, a biologically active macromolecular conjugate is provided of a biologically active polypeptide or glycopolypeptide and one or more water-soluble polymer molecules covalently bonded thereto at a reactive carbonyl or carboxylic acid group of a peptide moiety on the polypeptide or glycopolypeptide by a linkage containing a hydrazide or hydrazone functional group. The linkage is formed by reacting an acyl hydrazine derivative of the water-soluble polymer with a polypeptide or glycopolypeptide having an activated carboxylic acid group or a reactive carbonyl group generated thereon.

The present invention also provides a biologically active macromolecular conjugate of a biologically active glycopolypeptide and one or more water-soluble polymer molecules covalently bonded thereto at an oxidized carbohydrate moiety of the glycopolypeptide by a linkage containing a hydrazide or hydrazone functional group bound to the polymer via a short peptide sequence. The oxidation of the carbohydrate moiety produces reactive aldehydes. The hydrazone linkage is formed by reacting an acyl hydrazine derivative of the water-soluble polymer

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containing the peptide sequence with these aldehyde groups. The hydrazone can be further stabilized by reduction to a very stable alkyl hydrazine derivative.

5 The peptide sequence influences the lability of the linkage to proteolytic enzymes and also allows convenient characterization of the polymer conjugates by amino acid analysis of their hydrolysates. By using state-of-the-art techniques of amino acid analysis, the quantity of peptide sequences, and consequently the
10 degree of conjugation, can be determined for picomolar concentrations of the conjugate.

Therefore, it is also in accordance with the present invention that the peptide sequences also be utilized with the polypeptide conjugates of the present
15 invention to bind the linkages containing a hydrazide or hydrazone functional group to the water-soluble polymer.

Brief Description of the Drawings

FIG. 1 is a GF-HPLC chromatogram comparison of mPEG-beta-alanine-bovine serum albumin conjugate to
20 native bovine serum albumin.

FIG. 2 is a GF-HPLC chromatogram comparison of mPEG-beta-alanine-ovalbumin conjugate to native ovalbumin.

FIG. 3 is a GF-HPLC chromatogram comparison of
25 PEG-beta-alanine-IgG, conjugated via oxidized carbohydrate moieties, to native IgG.

FIG. 4 is a GF-HPLC chromatogram comparison of PEG-beta-alanine-rhG-CSF, conjugated via carboxylic acid groups of rhG-CSF, to native rhG-CSF.

Best Mode of Carrying Out the Invention

30

The macromolecules of the present invention are biologically active polypeptides or glycopolypeptides having one or more water-soluble polymer molecules covalently bonded thereto. The term
35 "biologically active" is used consistently with the meaning commonly understood to those of ordinary skill in the polypeptide and glycopolypeptide art, which meaning is not limited to physiologically or

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pharmacologically activities of the polypeptides or glycopolypeptides in the therapeutic sense. For example, many physiologically active polypeptides such as enzymes, the water-soluble polymer conjugates of which have therapeutic applications, are also able to catalyze reactions in organic solvents. Likewise, while therapeutic uses exist for water-soluble polymer conjugates of proteins such as concanavalin A, immunoglobulins, and the like, the polymer conjugates of these proteins are also useful as laboratory diagnostic tools.

Enzymes of interest, for both biological applications in general and therapeutic applications in particular include the oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases disclosed by U.S. Patent No. 4,179,337, the disclosure of which is hereby incorporated herein by reference thereto. Without being limited to particular enzymes, examples of specific enzymes of interest include asparaginase, arginase, adenosine deaminase, superoxide dismutase, catalase, chymotrypsin, lipase, uricase and bilirubin oxidase. Carbohydrate-specific enzymes are also of interest--for example, glucose oxidase, glucosidase, galactosidase, glucocerebrosidase, glucuronidase, etc.

Examples of other proteins of general biological or therapeutic interest include, but are not limited to, Factor VIII and polypeptide hormones such as insulin, ACTH, glucagon, somatostatin, somatotropins, thymosin, parathyroid hormone, pigmentary hormones, somatomedins, erythropoietin, luteinizing hormone, hypothamic releasing factors, antidiuretic hormones and prolactin.

Examples of glycopolypeptides of interest include, but are not limited to, immunoglobulins, chorionic gonadotrophin, follicle-stimulating hormone, thyroid-stimulating hormone, ovalbumin, bovine serum albumin (BSA), lectins, tissue plasminogen activator, numerous enzymes and glycosilated interleukins,

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interferons and colony stimulating factors. Immunoglobulins of interest include IgG, IgE, IgM, IgA, IgD and fragments thereof.

Many of the above glycopolypeptides such as the interleukins, interferons and colony stimulating factors also exist in non-glycosilated form, usually the result of preparation by recombinant protein techniques. The structure of such versions may not contain carbohydrate moieties. However, the non-glycosilated versions are still capable of conjugation at reactive carbonyl or carboxylic acid groups of the peptide moieties.

Examples of allergen proteins and glycoproteins having reduced allergenicity when conjugated with water-soluble polymers and consequently suitable for use as tolerance inducers include those allergens disclosed by Dreborg et al., Crit. Rev. Therap. Drug Carrier Syst., discussed above, the teachings of which are hereby incorporated herein by reference thereto. Among the allergens disclosed by this article are Ragweed Antigen E, honey bee venom, mite allergen, and the like.

The water-soluble polymers suitable for attachment to the polypeptides and glycopolypeptide include polyalkylene oxides, polyoxyethylenated polyols, polyacrylamides, polyvinyl pyrrolidone, polyvinyl alcohol, dextran, and other carbohydrate-based polymers. To be suitable for use in the present invention, the polymer must be soluble in water at room temperature. Polyalkylene oxide homopolymers meeting this requirement are polyethylene glycol (PEG) and copolymers thereof. Block copolymers of PEG with polypropylene glycol or polypropylene oxide are also suitable for use with the present invention, provided that the degree of block copolymerization is not so great as to render the polymer insoluble in water at room temperature. Examples of polyoxyethylenated polyols include

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polyoxyethylenated glycerols, polyoxyethylenated sorbitols, polyoxyethylenated glucoses, and the like.

The molecular weight of the polymer is not critical, and will depend mainly upon the end use of a particular polymer conjugate. Those of ordinary skill in the art are capable of determining molecular weight ranges suitable for their end use applications. In general, the useful range of molecular weight is a number average molecular weight between about 600 and about 100,000 daltons, and preferably between about 2,000 and about 20,000 daltons.

One or more polymer units can be attached covalently to the polypeptide or glycopolypeptide by reacting an acyl hydrazine derivative of the polymer with a polypeptide or glycopolypeptide having a reactive carbonyl group or an activated peptide carboxylic acid group. For purposes of the present invention, the reactive carbonyl group is defined as being either a ketone or aldehyde group, excluding other carboxyl-containing groups such as amides. Aldehyde groups are preferred, because they are more reactive than ketones.

The carbonyl group can be generated either on a peptide or a saccharide unit. For example, Dixon, J. Protein Chem., 3, 99 (1984) has reviewed some of the methods to generate reactive carbonyl groups on the N-terminus of a polypeptide molecule. Carbonyl groups can be generated on peptides, for example, by reacting a polypeptide or glycopolypeptide with a suitable heterobifunctional reagent such as a reactive ester of formyl benzoic acid, disclosed by King et al., Biochemistry, 25, 5774 (1986), the teachings of which are hereby incorporated herein by reference thereto. Carbonyl groups can be generated on saccharide units of glycopolypeptides, for example, by oxidizing vicinal diols of carbohydrate moieties of glycopolypeptides with excess periodate or enzymatically e.g. by use of galactose oxidase.

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The polymer acyl hydrazine reacts with the reactive carbonyl group on the polypeptide or glycopolypeptide to form a hydrazone linkage between the polymer and the polypeptide or glycopolypeptide. The
5 hydrazone can be reduced to a more stable alkyl hydrazide by using for example NaBH_4 or NaCNBH_3 .

The activated peptide carboxylic acid group can be derived either from a C-terminus carboxylic acid group or a carboxylic acid group of aspartic or glutamic
10 acid residues. The polymer acyl hydrazine reacts with the activated peptide carboxylic acid group to form a diacylhydrazine linkage between the polymer and the polypeptide or glycopolypeptide.

Activated carboxylic acid groups are carboxylic acid groups substituted with a suitable leaving group capable of being displaced by the polymer acyl hydrazine. Examples of suitable leaving groups are disclosed by Bodanszky, Principles of Peptide Synthesis
15 (Springer-Verlag, New York, 1984), the disclosure of which is hereby incorporated herein by reference thereto. Such leaving groups, which are well-known in the art of peptide chemistry, include, but are not limited to, imidazolyl, triazolyl, N-hydroxysuccinimidyl, N-hydroxynorbornenedicarboximidyl and phenolic
20 leaving groups, and are substituted onto the peptide carboxylic acid group by reacting the polypeptide or glycopolypeptide in the presence of an activating reagent with the corresponding imidazole, triazole, N-hydroxysuccinimide, N-hydroxynorbornene dicarboximide
25 and phenolic compounds.
30

Suitable activating reagents are also well-known and disclosed by the above-cited Bodanszky, Principles of Peptide Synthesis, the disclosure of which is hereby incorporated herein by reference thereto.
35 Examples of such activating reagents include, but are not limited to, water-soluble carbodiimides such as ethyl dimethylamino-propyl carbodiimide (EDC) and 3-[2-morpholinyl-(4)-ethyl] carbodiimide, p-toluen

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sulfonate, 5-substituted isoxazolium salts, such as Woodward's Reagent K, and the like.

The acyl hydrazine polymer derivatives of the present invention will have the general structure (I):



wherein R is one of the above-disclosed water-soluble polymers, Z is O, NH, S or a lower alkyl group containing up to ten carbon atoms, and X is a terminal group on the polymer. X can be a hydroxyl group, in which case the polymer has two labile groups per polymer moiety capable of reacting to form a derivative that can be covalently linked with a polypeptide or glycopolypeptide. X can therefore also be a group into which the terminal hydroxyl group may be converted, including the reactive derivatives of the prior art disclosed in U.S. Patent Nos. 4,179,337 and 4,847,325, the disclosures of which are hereby incorporated herein by reference thereto, as well as the acyl hydrazine derivatives of the present invention. Such heterobifunctional polymers can be prepared by methods known to those skilled in the art, including the methods disclosed by the present specification with reference to the preparation of acyl hydrazine derivatives, as well as the methods disclosed by Zalipsky and Barany, Polym. Prepr., 27(1), 1 (1986) and Zalipsky and Barany, J. Bioact. Compat. Polym., 5, 227 (1990), the disclosures of which are hereby incorporated herein by reference thereto.

30 Where X is a functional group useful for covalently linking the polymer with a second polypeptide or glycopolypeptide, X can be a solid support or a small molecule such as a drug, or an acyl hydrazide derivative of the formula (II):



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When Z is the same as disclosed above for acyl hydrazine derivatives, the polymer will then be a symmetrical, homobifunctional polymer derivative.

Such double polymer substitution can result in either intra- or intermolecular crosslinking of the polypeptide and glycopolypeptide moieties, which, in some cases, can be useful. Such crosslinking can be controlled by the amount of polymer used and the concentration of reacting species, which methods are well-known to those of ordinary skill in the art.

Crosslinking of the polypeptide or glycopolypeptide moieties can also be prevented by using a pre-blocked polymer having only one labile hydroxyl group per polymer moiety. With such polymers, X would represent a blocking group such as an alkoxy group of one to four carbon atoms. The preferred blocking group is a methoxy group.

In any event, the selectivity of the acyl hydrazines for the reactive carbonyl or activated carboxylic acid groups over the peptide amino group prevents intermolecular crosslinking between peptide amino groups and the reactive carbonyl groups and activated carboxylic acid groups, limiting occurrences of such crosslinking to instances when bifunctional polymer derivatives are employed.

X can also represent an antibody or solid support covalently coupled to the polymer by methods known to those skilled in the art. Examples of solid supports covalently coupled to water-soluble polymers and methods of coupling water-soluble polymers to solid supports are disclosed in Published European Patent Application No. 295,073, the disclosure of which is hereby incorporated herein by reference thereto.

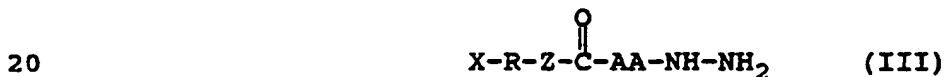
The acyl hydrazine derivative is prepared by reacting, for example, the terminal -OH group of methoxylated PEG (mPEG-OH) with phosgene to form mPEG-chloroformate as described in U.S. Patent Appln. Ser. No. 340,928 by Zalipsky, filed April 19, 1989, the

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disclosure of which is hereby incorporated herein by reference thereto. The reaction is carried out in organic solvents in which the reactants are soluble, such as methylene chloride, and will run to completion overnight at room temperature. The solvents and excess phosgene are removed and the residue of polymeric chloroformate is then reacted with an excess of hydrazine.

The preparation of acyl hydrazine polymer derivatives is described with reference to mPEG for purposes of illustration, not limitation. Similar products would be obtained with any of the polymers suitable for use with the present invention, and it will be clear to those of ordinary skill in the art how this preparation can be adapted to the other suitable polymers.

A more preferred form of the present invention uses polymer hydrazides of the general formula (III):



wherein R represents the water-soluble polymers, Z represents the groups described above with respect to Formula I, X represents the polymer terminal groups described above and AA represents an amino acid or a peptide sequence. AA can be a peptide sequence of any of the common amino acids, or at least one amino acid residue. In the case of AA being one amino acid residue, it is preferable that it is a residue that does not appear naturally in proteins. Examples of such unusual residues include, but are not limited to, alpha- or gamma- amino butyric acid, norleucine, homoserine, beta-alanine, epsilon-caproic acid, and the like.

When Z is oxygen, the linkage is a urethane linkage, which is very stable at ambient temperature in a variety of buffers, even at extreme pH's, but is readily split under conditions normally used for protein hydrolysis, thus allowing determination of amino acid components of AA by amino acid analysis.

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The peptide sequence can serve two roles. First, it can provide for convenient characterization of the modified protein by quantitation of the sequence by amino acid analysis. In this instance, the peptide sequence preferably is as short as possible and preferably contains unusual amino acid residues. For characterization of the modified protein, the peptide sequence most preferably contains but one amino acid.

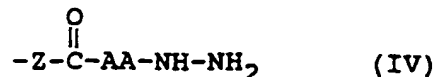
In addition, AA can also contain a labeled amino acid residue (chromophore, fluorophore, or radioisotope containing), or an amino acid that could be easily labeled (e.g. tyrosine can be iodinated). The presence of such labels would facilitate the experimental evaluation of the resulting polymer-polypeptide conjugates.

Second, the peptide sequence can optimize the lability of the covalent linkage between the water-soluble polymer and the polypeptide to proteolytic enzymes. In this second instance, the peptide sequence is preferably as long as possible and preferably contains natural amino acid residues. By controlling enzymatic lability in this manner, the polymer conjugates can be used to deliver physiologically active polypeptides or glycopolypeptides to specific sites, such as cancer cells having elevated concentrations of certain proteolytic enzymes to which the peptide sequence is labile.

The length and sequence of the peptide in this second instance can be fine-tuned depending on the system of use and specificity of the target enzyme. Usually, three to seven amino acid residues would be required. Using modern techniques of peptide chemistry such short peptide sequences can be readily assembled.

In symmetrical, homobifunctional polymer derivatives, X can also contain a second peptide sequence residue. For example, when X is an acyl hydrazine derivative, X would have the general formula (IV):

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wherein Z and AA are as described above.

The acyl hydrazine polymer derivative
5 containing a peptide sequence can be synthesized by
first preparing the polymeric chloroformate as described
above. The polymeric chloroformate is then reacted with
the peptide or an amino acid derivative in a solvent in
10 which the polymeric chloroformate is soluble, such as
methylene chloride. The peptide or amino acid is
preferably in the form of the ester of the C-terminus
acid group, more preferably methyl or ethyl esters.

This reaction is also operative under mild
conditions and typically runs to completion at room
15 temperature and the resulting product can be readily
converted to a hydrazide by hydrazinolysis. The acyl
hydrazine polymer derivative containing a peptide
sequence is then recovered and purified by conventional
methods, such as repeated precipitation of the polymer-
20 product.

Alternatively, the acyl hydrazine polymer
derivative containing a peptide sequence or an amino
acid can be prepared by reacting the peptide sequence
with a succinimidyl carbonate active ester of the
25 polymer, as disclosed by the above-mentioned Zalipsky,
U.S. Patent Appln. No. 340,928 or by directly reacting
isocyanate derivatives of an amino acid with the
terminal hydroxyl group of the polymer as disclosed by
Zalipsky et al., Int. J. Peptide Protein Res., 30, 740
30 (1987), the disclosures of both of which are hereby
incorporated herein by reference thereto. Again, both
reactions are essentially conventional and operative
under mild conditions, running to completion at room
temperature in organic solvents in which the polymer is
35 solvent, such as methylene chlorid. The reaction of
isocyanate derivatives of amino acid esters with
terminal hydroxyl groups of polymers is disclosed in the
above-cited Zalipsky and Barany, Polym. Prepr., as well

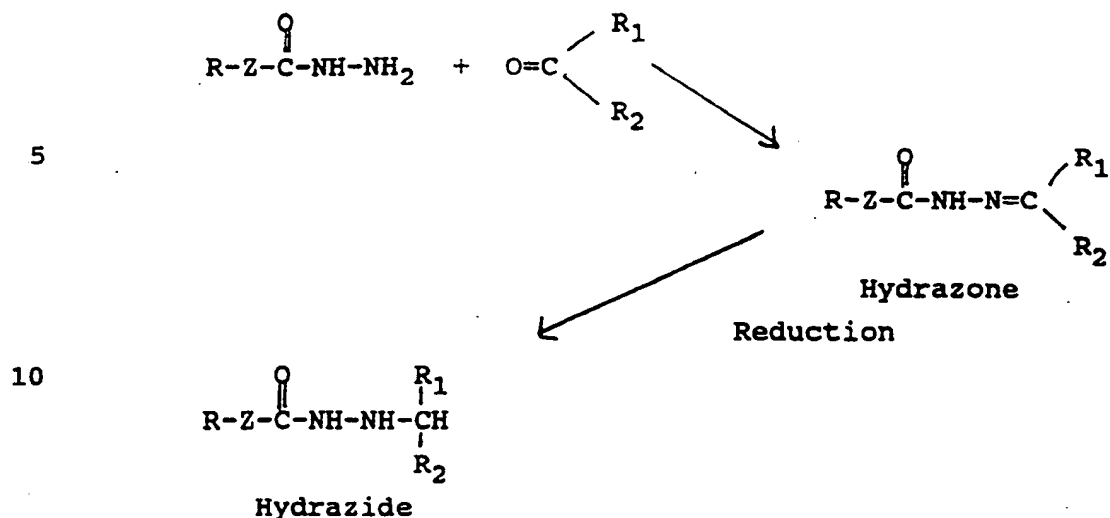
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as in Zalipsky et al., Int. J. Peptide Protein Res., the teachings of both of which are hereby incorporated herein by reference thereto. The succinimidyl carbonate derivative of the polymer is formed by the known method of reacting the above-disclosed polymeric chloroformate with N-hydroxysuccinimide, as disclosed by the above-cited Zalipsky, U.S. Patent Appln. No. 340,928, the disclosure of which is hereby incorporated herein by reference thereto.

10 Either of the above polymer-polypeptide derivatives can be readily converted to a hydrazide by the hydrazinolysis method disclosed above to yield an acyl hydrazine. The preparation of peptide sequences is essentially conventional and disclosed by the above-cited Bodanszky, Principles of Peptide Synthesis, the disclosure of which is hereby incorporated herein by reference thereto.

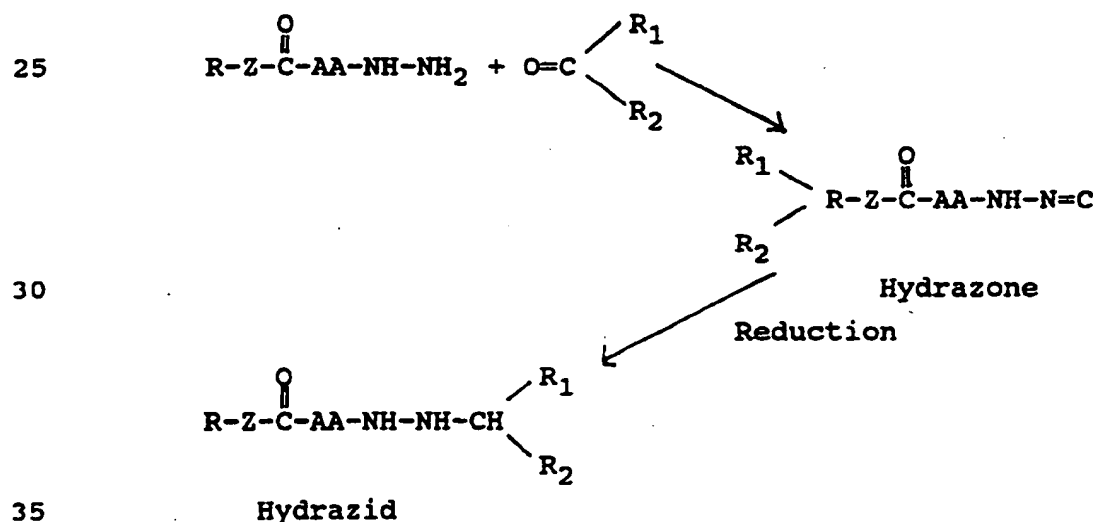
20 The reaction of polymer acyl hydrazine derivatives with carbonyl-containing polypeptides and glycopolypeptides to form a hydrazone linkage is illustrated by the reaction sequence of Scheme 1 in which R represents the above-described water-soluble polymers, Z is as described above with respect to Formulae I-IV and either or both of R_1 and R_2 are independently selected from oxidized carbohydrate moieties of glycopolypeptides and peptide units of polypeptides and glycopolypeptides on which reactive carbonyl groups have been generated:

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Scheme 1

15 The hydrazone can be reduced to the more stable alkyl hydrazide by reacting the hydrazone with, for example, NaBH_4 or NaCNBH_3 .

20 The reaction of polymer acyl hydrazine derivatives containing peptide sequences, with carbonyl-containing polypeptides and glycopolypeptides is shown in Scheme 1A, in which R, R_1 , R_2 and Z are the same as described above with respect to Scheme 1 and AA represents the above-described peptide sequence:

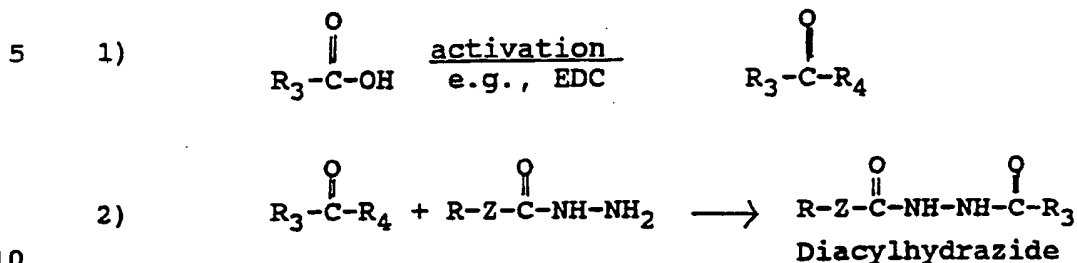
Scheme 1A

The reaction of polymer acyl hydrazine derivatives with activated peptide carboxylic acid groups of polypeptides and glycopolypeptides to form

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diacylhydrazides is illustrated by the reaction sequence of Scheme 2:

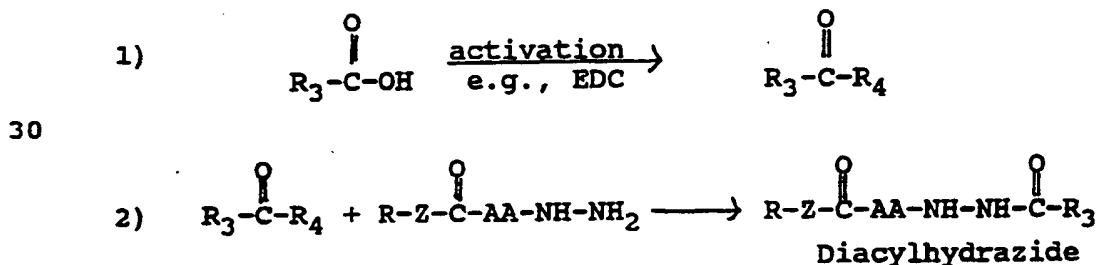
Scheme 2



R again represents the above-described water-soluble polymers, and Z is the same as described above for Formulae I-IV. R₃ represents a polypeptide containing aspartic acid, glutamic acid or a C-terminus carboxylic acid residues. R₄ represents one of the above-described leaving groups substituted on the peptide carboxylic acid when the carboxylic acid group is activated as described above.

The reaction of polymer acyl hydrazine derivatives containing peptide sequences, with activated peptide carboxylic acid groups of polypeptides and glycopolypeptides is shown in Scheme 2A, in which R, R₃, R₄ and Z are the same as described above with respect to Scheme 2, and AA represents the above-described peptide sequence:

Scheme 2A



Generally, the conjugation of a polypeptide or glycopolypeptide with a water-soluble polymer first involves either oxidizing carbohydrate moieties of the glycopolypeptide or activating carboxylic acid groups of peptide moieties of the polypeptides or glycopolypeptides. The carbohydrate moieties can be

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oxidized by reacting the glycopolypeptide in aqueous solution with sodium periodate or enzymatically using galactose oxidase or combination of neuraminidase and galactose oxidase as disclosed by Solomon et al.,
5 J. Chromatography, 510, 321-9 (1990). The reaction runs rapidly to completion at room temperature. The reaction medium is preferably buffered, depending upon the requirements of the polypeptide or glycopolypeptide. The oxidized glycopolypeptide is then recovered and
10 separated from the excess periodate by column chromatography.

Carboxylic acid groups of peptide moieties can be activated by reacting the polypeptide or glycopolypeptide with an activating reagent such as a
15 water-soluble carbodimide such as EDC. The reactants are contacted in an aqueous reaction medium at a pH between about 3.0 and 8.0, and preferably about 5.0, which medium may be buffered to maintain the pH. This reaction is taking place under mild conditions
20 (typically 4 to 37°C) that are tolerated well by most proteins.

Polypeptides or glycopolypeptides having peptide units on which reactive carbonyl groups have been generated may be directly reacted with the acyl
25 hydrazine polymer derivatives in an aqueous reaction medium. This reaction medium may also be buffered, depending upon the pH requirements of the polypeptide or glycopolypeptide and the optimum pH for the reaction, which pH is generally between about 5.0 and about 7.0
30 and preferably about 6.0.

In all instances, the optimum reaction media pH for the stability of particular polypeptides or glycopolypeptides and for reaction efficiency, and the buffer in which this can be achieved, is readily
35 determined within the above ranges by those of ordinary skill in the art without undue experimentation. For purposes of this application, the operativeness of the within reactions under mild conditions is defined as

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meaning that the preferred temperature range is between about 4 and about 37°C. Those of ordinary skill in the art will understand that the reactions will run somewhat faster to completion at higher temperatures, with the proviso that the temperature of the reaction medium cannot exceed the temperature at which the polypeptides or glycopolypeptides begin to denature. Furthermore, those of ordinary skill in the art will understand that certain polypeptides and glycopolypeptides will require reaction with the polymer acyl hydrazine derivatives at reduced temperatures to minimize loss of activity and/or prevent denaturing. The reduced temperature required by particular polypeptides and glycopolypeptides is preferably no lower than 4°C and in no event should this temperature be lower than 0°C. The reaction will still take place, although longer reaction times may be necessary.

Usually, the polypeptide or glycopolypeptide is reacted in aqueous solution with a quantity of the acyl hydrazine polymer derivative in excess of the desired degree of conjugation. This reaction also proceeds under mild conditions, typically at 4 to 37°C. The reaction medium may be optionally buffered, depending upon the requirements of the polypeptide or the glycopolypeptide, and the optimum pH at which the reaction takes place. Following the reaction, the conjugated product is recovered and purified by diafiltration, column chromatography or the like. When the acyl hydrazine polymer derivative includes an amino acid or a peptide sequence, the degree of polymer conjugation of the polypeptide or glycopolypeptide can then be determined by amino acid analysis.

In view of the foregoing, it can be readily appreciated that the acyl hydrazine polymer derivatives of the present invention possess the optimum balance of reactivity and selectivity so that polymer conjugates can be formed with non-amino functional groups of polypeptides and glycopolypeptides with virtually no

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competition between the acyl hydrazines and the peptide amino groups for the non-amino functional groups. Thus, crosslinking is prevented and the activity of the polypeptide or glycopolypeptide is preserved.

5 The following non-limiting examples set forth hereinbelow illustrates certain aspects of the invention. All parts and percentages are by weight unless otherwise noted, and all temperatures are in degrees Celsius.

10

EXPERIMENTAL

MATERIALS:

 Methoxy-PEG (mPEG) is available from Union Carbide. The solvents used, as well as beta-alanine ethyl ester HCL, hydrazine, P_2O_5 , EDC, 15 N-hydroxy-5-norbornene-2,3-dicarboximide (HONb), $NaCNBH_3$ and $NaIO_4$ are available from Aldrich Chemicals of Milwaukee, Wisconsin. Chymotrypsin was obtained from Worthington Chemical. BSA, ovalbumin and human immunoglobulin G (IgG) are available from Sigma Chemical 20 of St. Louis, Missouri. G-CSF was obtained from Amgen of Thousand Oaks, California.

EXAMPLE 1

SYNTHESIS OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE:

25 mPEG (MW_n 5,000, 100 g, 20 mmol) was dissolved in toluene (250 mL) and azeotropically dried for two hours under reflux. The solution was brought to 25°C, diluted with methylene chloride (50 mL) and then treated with phosgene (30 mL of 20 percent toluene 30 solution, 56 mmol) overnight. The solvents and the excess of phosgene were removed by rotary evaporation under vacuum. The solid residue of polymeric chloroformate was dissolved in methylene chloride (90 mL) and treated with beta-alanine ethyl ester 35 hydrochloride (6.1 g, 40 mmol) predissolved in methylene chloride (total volume 30 mL) followed by triethylamine (8.4 mL, 60 mmol). Approximately 30 minutes later, the solution was diluted with toluene (50 mL), filtered and

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evaporated to dryness. The crude product was dissolved in warm (50°C) ethyl acetate (500 mL) and filtered through celite. The filtrate was diluted with isopropanol to a total volume of 1,000 mL and left overnight at 25°C to facilitate precipitation of the product. Another recrystallization of the product from isopropanol was performed. The yield of dried mPEG-beta-alanine ethyl ester was 98 g (95%). The following IR and NMR spectrum were then obtained:

10 IR (neat): 3341 (N-H), 1723 (C=O, urethane) cm^{-1} . ^1H -NMR (CDCl_3): Δ 1.17 (t, $\text{CH}_3\text{CH}_2\text{O}$), 2.44 (t) CH_2CH_2 of beta-alanine), 3.64 (PEG), 3.9 (t, NH (C=O) OCH_2), 4.11 (2, $\text{CH}_3\text{CH}_2\text{O}$), 5.25 (broad, NH) ppm.

The mPEG-beta-alanine ethyl ester (62 g, 12 mmol) was dissolved in pyridine (120 mL) and treated with hydrazine (12 mL, 0.375 mole) under reflux for six hours. The solution was rotary evaporated to dryness and the residue crystallized twice from isopropanol and dried in vacuo over P_2O_5 . The yield was 60 g (97%).

20

The absence of free hydrazine in the product was ascertained by reverse-phase (C-18) thin-layer chromatography in water/methanol (3:1) using TNBS spraying solution for detection.

25 Colorimetric assay of hydrazide groups using TNBS gave 0.2 mmol/g (103% of theoretical). The beta-alanine content of the polymer was 0.205 mmol/g (105% of theoretical) as determined by amino acid analysis of a completely hydrolysed

30 (6N HCl, 110°C, 24 h) aliquot of the product. ^{13}C -NMR (CDCl_3): Δ 171.2 (C=O, hydrazide); 156.4 (C=O, urethane); 71.8 (CH_3OCH_2); 70.0 (PEG); 68.5 ($\text{CH}_2\text{CH}_2\text{OC=O}$); 63.7 ($\text{CH}_2\text{CH}_2\text{OC=O}$); 58.9 (CH_3O); 37.1 (NHCH_2CH_2); 33.9 (NHCH_2CH_2) ppm. IR (neat): 3328 (NH); 1719 (C=O, urethane); 1671 (C=O, hydrazide) cm^{-1} .

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EXAMPLE 2COUPLING OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO EDC-ACTIVATED CARBOXYL GROUPS OF CHYMOTRYPSIN:

5 Chymotrypsin (20 mg, 8.0×10^{-7} mole,
1.28 $\times 10^{-5}$ equiv. of carboxyl) and the
mPEG-beta-alanine-hydrazide derivative of Example 1
(800 mg, 0.16 mmol) were dissolved in 8 ml of 1 mM HCl,
10 the solution was brought to pH 5.0 and treated with EDC
(15 mg, 0.078 mmol). The reaction mixture was stirred
gently at 25°C overnight while pH 5.0 was maintained by
addition of 1.0 N HCl. Excess reagents were removed by
extensive diafiltration of the reaction solution at 4°C
15 against one mM HCl. In order to determine the extent of
the coupling reaction, an aliquot of the
PEG-chymotrypsin conjugate was completely hydrolyzed
(6 N HCl, 110°C, 24 hours) and amino acid analysis was
performed. The amount of beta-alanine corresponded
to 2.4 molecules of mPEG per molecule of chymotrypsin.

20

EXAMPLE 3COUPLING OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO HONb ACTIVATED CARBOXYL GROUPS OF CHYMOTRYPSIN:

The same conjugation protocol as Example 2 was
25 employed, in the presence of HONb (28.7 mg, 0.16 mmol).
The PEG-chymotrypsin obtained had an
average 2.7 molecules of mPEG per molecule of protein,
based on quantitation of beta-alanine by amino acid
analysis. This demonstrates that the conjugation
30 process is only slightly enhanced by the presence of
HONb.

EXAMPLE 4COUPLING OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO EDC-ACTIVATED CARBOXYL GROUPS OF BSA:

35 A solution of BSA (20 mg) and a
mPEG-beta-alanine hydrazide derivative of Example 1
(800 mg, 0.16 mmol) in 50 mM NaCl (10 mL) was treated
with EDC (15 mg, 0.078 mmol) overnight at pH 5.0, 25°C

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as in Example 2. Excess reagents were removed by extensive diafiltration of the reaction solution at 4°C against phosphate buffer (50 mM, pH 7.7). The content of beta-alanine in the conjugate corresponded to 8.1 residues of mPEG per molecule of BSA. A GF-HPLC comparison of the PEG-conjugate to native BSA was performed with a BIOSEP SEC 4000 column, the results of which are depicted in FIG. 1. The elution conditions were 10% (vol/vol) methanol/40 mM phosphate buffer. FIG. 1 depicts good homogeneity of the PEG-conjugate 1, with a substantially increased molecular weight as compared to the native BSA 2.

EXAMPLE 5

COUPLING OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO OXIDIZED CARBOHYDRATE MOIETIES OF OVALBUMIN:

Ovalbumin (20 mg, 4.4×10^{-7} mole) dissolved in Phosphate Buffered Saline (PBS) buffer, pH 6.0 (1.8 mL) was treated with NaIO_4 (0.2 mL of 200 mM aqueous solution). The reaction was allowed to proceed in the dark at 4°C. After one hour, the oxidized glycoprotein was separated from the excess of periodate by passing the reaction solution through a 12 mL Sephadex G-25 column equilibrated with acetate buffer to pH 5.0. Additional samples were prepared and the procedure was repeated equilibrating the column with PBS buffer at pH 6.0 and phosphate buffer at pH 7.0. This resulted in three separate reaction mixtures having different buffering systems. To each mixture was added the mPEG-beta-alanine-hydrazide derivative of Example 1 (150 mg, 2.9×10^{-5} mole). Each of the three reaction mixtures was divided into two equal portions and NaCNBH_3 (0.3 mL of 6.6 mg/mL solution, 3.15×10^{-5} mole) was added to one portion of each. The reactions were allowed to proceed overnight at 4°C. Each solution was diafiltered using phosphate buffer pH 7.7 until all the unreacted reagents were removed. The conjugates in the solutions to which the NaCNBH_3 was added formed

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acylhydrazine linkages. Analyses of the mPEG-ovalbumin conjugates are summarized in Table I below.

Table I			
pH	Buffer	Type of Linkage	# of mPEG in Conjugate*
5.0	acetate	hydrazone	3.8
5.0	acetate	acylhydrazine	3.6
6.0	PBS	hydrazone	4.3
6.0	PBS	acylhydrazine	2.4
7.0	phosphate	hydrazone	3.1
7.0	phosphate	acylhydrazine	3.0

* The average number of mPEG chains attached to an ovalbumin molecule was calculated from the results of amino acid analysis of the conjugates.

Depicted in FIG. 2 is the GF-HPLC analysis using a TSK G 4000SW column and a 10% (vol/vol) methanol/40 mM phosphate buffer pH 7.5 mobile phase, which showed good homogeneity of the mPEG-ovalbumin conjugate 3, and a substantially increased molecular weight as compared to the native ovalbumin 4.

EXAMPLE 6

ATTACHMENT OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO THE CARBOHYDRATE MOIETY OF IMMUNOGLOBULIN G:

Human immunoglobulin G (IgG) (5 mg, 3.12×10^{-5} mmol) in PBS (0.8 mL, 50 mM, pH 6.0) was treated with a freshly prepared solution of sodium periodate (0.2 mL, 200 mM) in PBS. The resulting solution was incubated at 4°C. After one hour, the oxidized glycoprotein was separated from the excessive periodate by passing the reaction solution through a 12 mL Sephadex G-25 column. The oxidized IgG was collected and treated with the mPEG-beta-alanine hydrazide derivative of Example 1 (200 mg, 1.25×10^{-3} mmol) at 4°C overnight. Each solution was diafiltered using phosphate buffer pH 7.7

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until all unreacted reagents were removed. A GF-HPLC comparison of the conjugate to native IgG was performed with a ZORBAX GF-450 column, the results of which are depicted in FIG. 3. A 0.2 M phosphate buffer, pH 7.5 mobile phase was used. FIG. 3 depicts good homogeneity of the PEG-conjugate 5, with a substantially increased molecular weight as compared to the native IgG 6. The amount of beta-alanine was determined by amino acid analysis of a hydrolyzed (6 N HCl, 110°C, 24 h) aliquot of the PEG-IgG conjugate to correspond to six residues of mPEG per protein molecule.

EXAMPLE 7

ATTACHMENT OF mPEG-HYDRAZIDE DERIVATIVE CONTAINING BETA-ALANINE TO THE CARBOHYDRATE MOIETY OF IMMUNOGLOBULIN G WITHOUT REMOVAL OF EXCESS PERIODATE:

IgG (5.4 mg, 3.37×10^{-5} mmol) and PBS (50 mM, 0.91 mL) was treated with a freshly prepared solution of sodium periodate (0.09 mL of 110 mM) at 4°C in the dark. After one hour, mPEG-beta-alanine hydrazide (100 mg, 6.3×10^{-4} mmol) was added to the reaction mixture, which was then incubated overnight at 4°C. The solution was diafiltered against phosphate buffer at pH 7.7 until all the unreacted reagents were removed. Pure PEG-IgG was obtained, which was determined by amino acid analysis of the beta-alanine content of a hydrolyzed aliquot of the conjugate (6 N HCl, 110°C, 24 h) to contain 8.6 residues of mPEG per molecule of IgG.

In addition to requiring fewer manipulations, it appears that this one-pot conjugation procedure is more efficient than the one described in EXAMPLE 6.

EXAMPLE 8

ATTACHMENT OF mPEG-HYDRAZIDE DERIVATIVE TO CARBODIIMIDE-ACTIVATED CARBOXYL GROUPS OF G-CSF:

The mPEG-beta-alanine-hydrazide of Example 1 (15.0 g, 2.9 mmol) was added to a solution of G-CSF (86 mg, 4.78×10^{-6} mole) in 1 mM HCl (86 mL), followed by EDC (128 mg, 0.667 mmol). The reaction mixture was

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gently stirred at 25°C for 90 minutes while maintaining the pH at about 4.7 to 5.0. Excess reagents were removed by extensive diafiltration of the reaction solution at 4°C against 1 mM HCl. A GF-HPLC comparison of the PEG-conjugate to native G-CSF was performed using a ZORBAX GF-450 column, the results of which are depicted in FIG. 4. The mobile phase was 0.2 M phosphate buffer pH 7.5. FIG. 4 depicts PEG-conjugate 7, with a substantially increased molecular weight as compared to native G-CSF 8.

The average number of mPEG residues in the PEG-G-CSF was 5.8, as determined by measuring the amount of beta-alanine in an hydrolyzed (6 N HCl, 110°C, 24 h) aliquot of the conjugate. TNBS assay confirmed that both native and PEG-modified G-CSF-1 had the same number of amino groups, indicating that the EDC activated carboxylic acid groups of the protein did not react with amino groups of the protein. The preparation of mPEG-G-CSF gave four separate bands on SDS-PAGE (PhastGel-, Homogenous 12.5, Pharmacia) in the range from 29,000 to 67,000 daltons. Isoelectric Focusing (PhastGel-, IEF 3-9, Pharmacia) of the mPEG-G-CSF-1 resulted in the separation of six bands with pI's arranging between 6.8 and 9.0, noticeably higher than the native protein (pI 5.2; 5.9). This clearly indicates that the protein became more basic as a result of the conjugation with the peptide carboxylic acid groups without crosslinking of the activated carboxylic acid groups with the peptide amino groups.

As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the spirit and scop of the invention, and all such modifications are intended to be included within the scop of the following claims.

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Industrial Applicability

The present invention is applicable to the production of polymers conjugated with various biologically active and pharmaceutically active compounds representing a novel form of drug delivery.

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Claims:

1. A biologically active macromolecular conjugate comprising a biologically active polypeptide or glycopolypeptide and one or more water-soluble
5 polymers covalently bonded thereto at a reactive carbonyl or carboxylic acid group of a peptide moiety on said polypeptide or glycopolypeptide by a linkage containing a hydrazide or hydrazone functional group.
2. The macromolecular conjugate of claim 1,
10 wherein said biologically active polypeptide or glycopolypeptide comprises a polypeptide.
3. The macromolecular conjugate of claim 2, wherein said polypeptide is an enzyme.
4. The macromolecular conjugate of claim 3,
15 wherein said enzyme is selected from the group consisting of asparaginase, arginase, adenosine deaminase, superoxide dismutase, catalase, chymotrypsin, lipase, uricase, bilirubin oxidase, glucose oxidase, glucosidase, galactosidase, glucocerebrosidase and
20 glucuronidase.
5. The macromolecular conjugate of claim 2, wherein said polypeptide is selected from the group consisting of Factor VIII, insulin, ACTH, glucagon, somatostatin, somatotropins, thymosin, parathyroid
25 hormone, pigmentary hormones, somatomedins, erythropoietin, luteinizing hormone, hypothalamic releasing factors, antidiuretic hormones, prolactin, interleukins, interferons and colony stimulating factors.
6. The macromolecular conjugate of claim 1,
30 wherein said biologically active polypeptide or glycopolypeptide comprises a glycopolypeptide selected from the group consisting of immunoglobulins, ovalbumin, lipase, glycocerebrosidase, lectins, tissue plasminogen
35 activator and glycosilated interleukins, interferons and colony stimulating factors.
7. The macromolecular conjugate of claim 1, wherein said linkage further includes a peptide sequence

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binding said hydrazide or hydrazone functional group to said polymer.

8. The macromolecular conjugate of claim 1, wherein said reactive carbonyl group is a ketone or aldehyde group generated on said peptide moiety.

9. A biologically active macromolecular conjugate comprising a biologically active glycopolypeptide and one or more water-soluble polymers covalently bonded thereto at an oxidized carbohydrate moiety of said glycopolypeptide by a linkage containing a hydrazide or hydrazone functional group bound to said polymer by a peptide sequence.

10. The macromolecular conjugate of claim 1 or claim 9, wherein said water-soluble polymer is selected from the group consisting of polyalkylene oxides, polyoxyethylenated polyols, polyvinyl alcohol, polyacrylamides, polyvinyl pyrrolidone and dextran.

11. The macromolecular conjugate of claim 10, wherein said polyalkylene oxide is a polyethylene glycol homopolymer.

12. The macromolecular conjugate of claim 11, wherein said polyethylene glycol homopolymer is a methoxylated polyethylene glycol homopolymer.

13. The macromolecular conjugate of claim 10, wherein said polyalkylene oxide is a block copolymer of polyethylene glycol with polypropylene glycol or polypropylene oxide.

14. The macromolecular conjugate of claim 10, wherein said polyoxyethylenated polyols are selected from the group consisting of polyoxyethylenated glycerols, polyoxyethylenated sorbitols and polyoxyethylenated glucoses.

15. The macromolecular conjugate of claim 1 or claim 9, wherein said water-soluble polymer has a number average molecular weight between about 600 and about 100,000 daltons.

16. The macromolecular conjugate of claim 15, wherein said water-soluble polymer has a number average

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molecular weight between about 2,000 and about 20,000 daltons.

17. The macromolecular conjugate of claim 9, wherein said glycopolyptide is selected from the group consisting of immunoglobulins, ovalbumin, lipase, glycocerebrosidase lectins, tissue plasminogen activator and glycosilated interleukins, interferons and colony stimulating factors.

18. The macromolecular conjugate of claim 6 or 7, wherein said immunoglobulin is selected from the group consisting of IgG, IgE, IgM, IgA, IgD and fragments thereof.

19. The macromolecular conjugate of claim 7 or 9, wherein said peptide sequence consists essentially of one amino acid.

20. The macromolecular conjugate of claim 7 or 9, wherein said peptide sequence comprises one or more amino acids that do not appear naturally in proteins.

21. The macromolecular conjugate of claim 20, wherein said amino acids are independently selected from the group consisting of alpha-amino butyric acid, gamma-amino butyric acid, norleucine, homoserine, beta-alanine and epsilon-caproic acid.

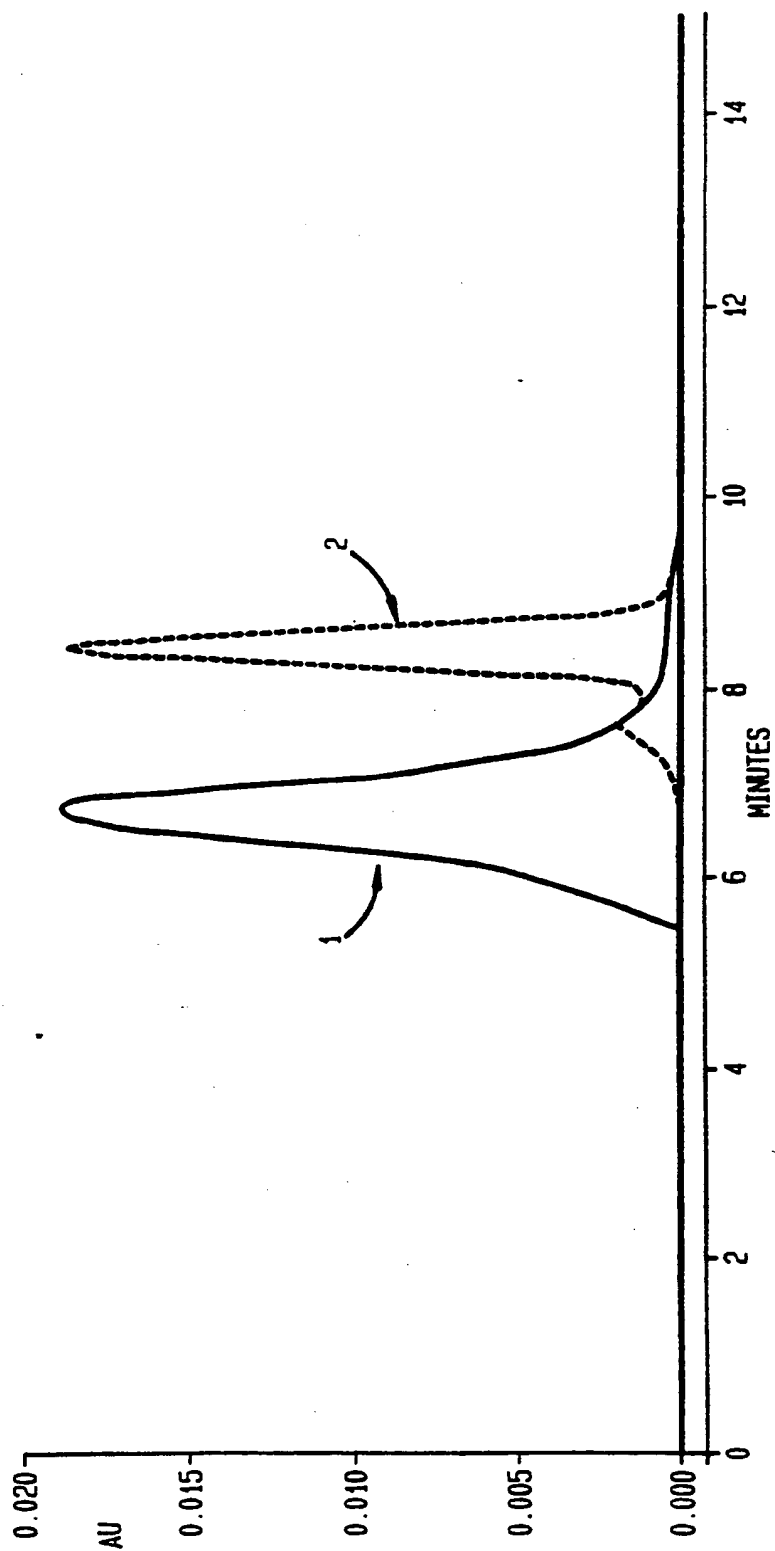
22. The macromolecular conjugate of claim 7 or 9, wherein said peptide sequence contains up to six amino acids.

23. The macromolecular conjugate of claim 7 or 9, wherein said amino acids occur naturally in proteins.

24. The macromolecular conjugate of claim 7 or 9, wherein said peptide sequence forms a urethane group with said polymer.

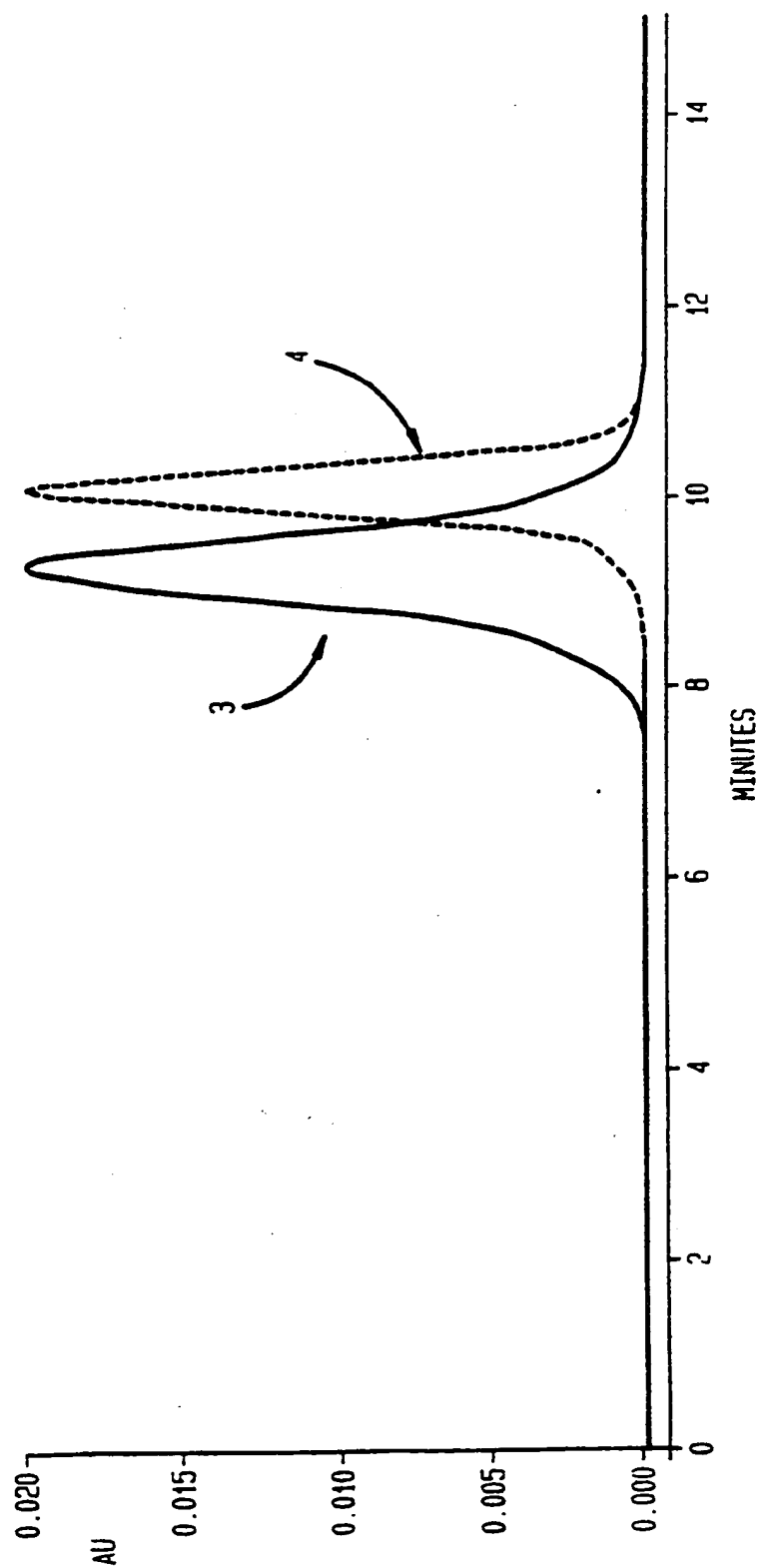
1 / 4

FIG. 1



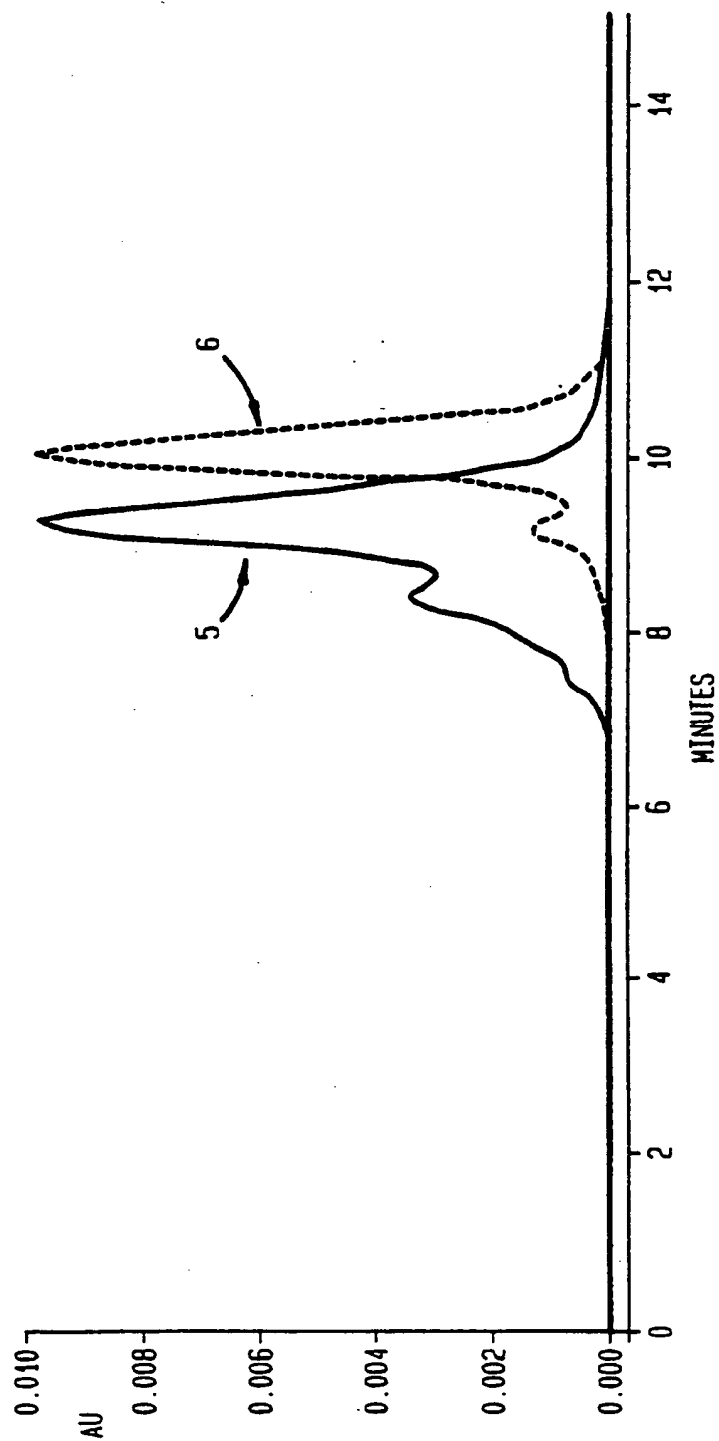
2 / 4

FIG. 2



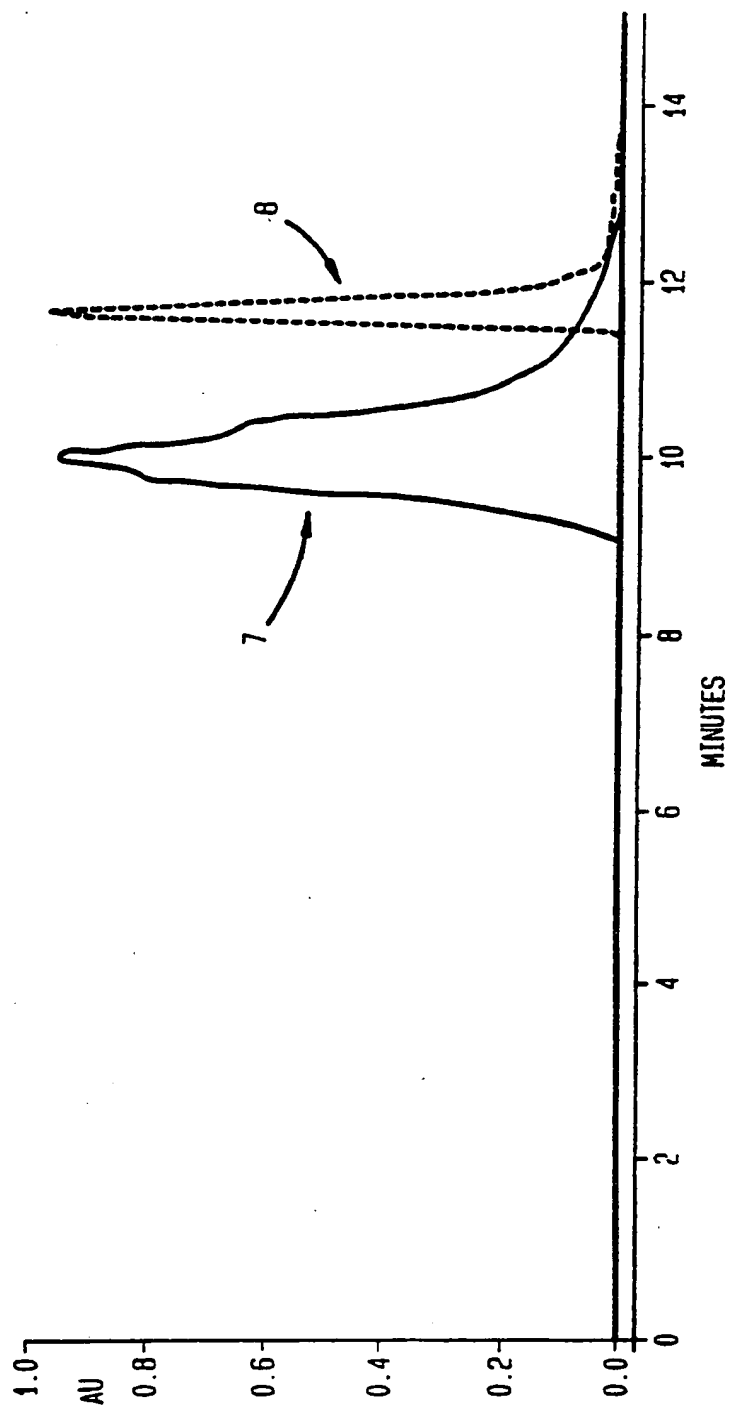
3 / 4

FIG. 3



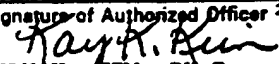
4 / 4

FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02047

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): Please See Attached Sheet.		
US CL : Please See Attached Sheet.		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	530/303, 306, 308, 311, 312, 350, 351, 367, 383, 391.1, 395, 396, 397, 399, 408, 409, 812, 813; 525/54.1; 435/188, 189, 190, 192, 198, 200, 212, 213, 215, 217, 961, 964	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
CAS online, File Registry, APS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category [*]	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X/Y	US, A, 4,847,325 (Shadle et al) 11 July 1989, see columns 2, 4, 9, 12 and 27.	1, 2, 3, 6, 8 - 1 2, 1 4 - 16/3, 4, 7, 13, 17 -24
Y	US, A, 4,766,106 (Katre et al) 23 August 1988, see columns 2, 3 and 7.	1-24
Y	US, A, 4,970,300 (Fulton et al) 13 November 1990, see columns 3, 4, 9 and 10.	1-24
Y	JP, A, 0399384 (Toyo Rubber Ind KK) 30 August 1978, see the Derwent abstract.	1-4, 6-17, 19-24
Y	Methods in Enzymology, Volume 138, issued 1987, M. Wilchek et al, "Labeling Glycoconjugates with Hydrazide Reagents", pages 429-442, see pages 434 and 435-440.	1-24
Y	Proceed. Intern. Symp. Control. Rel. Bioact. Mater., Volume 17, issued 1990, L. Sartore et al, "Soluble M-PEG with an aminoacid or peptide spacer arm and PVP for the modification of therapeutically usefull enzymes", pages 208-209, See the entire article.	1-24
<p>[*] Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (see specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
08 JUNE 1992	23 JUN 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	 KAY K. KIM, PH.D.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

Agric. Biol. Chem., Volume 52, No. 8, issued 1988, N. Yamasaki et al, "Novel Polyethylene Glycol Derivatives for Modification of Proteins", pages 2125-2127, see page 2125.

1-24

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:
2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C07K 7/26, 7/34, 7/40, 15/14, 15/26, 15/28, 17/08, 17/10; C08F 16/08, 20/18, 26/10;
C08L 29/04, 33/26, 39/06, 57/10; C12N 9/14, 9/68, 9/76, 9/82, 9/96, 11/06, 11/08,
11/10

I. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/303, 306, 308, 311, 312, 350, 351, 367, 383, 391.1, 395, 396, 397, 399, 408, 409;
525/54.1; 435/188, 189, 190, 192, 198, 200, 212, 213, 215, 217